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POLYPEPTIDE OF THE GROWTH FACTOR RECEPTOR FAMILY, APPLICATION TO THE DIAGNOSIS AND TREATMENT OF MYELOPROLIFERATIVE DISEASES

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POLYPEPTIDE OF THE GROWTH FACTOR RECEPTOR FAMILY, APPLICATION TO THE DIAGNOSIS AND TREATMENT OF MYELOPROLIFERATIVE DISEASES.

(57) Abstract

Polypeptide of a growth factor receptor family, having a specific sequence and presenting all or part of the following properties: it encourages and/or is involved in the proliferation and/or differenciation of hematopoietic cell lines when obtained from the MPLV retrovirus; it is capable of acting as a hematopoietic growth factor receptor; it is recognized by antibodies directed against it. Polypeptides similar to the above. Applications in the diagnosis of the expression of said ligand polypeptide.

Myeloproliferative diseases are diseases in which the hematopoietic stem cells present an alteration in their capacity for differentiation and/or an alteration in their dependency with regard to specific growth factor.

The blood cells come from a small number of stem cells capable of self-renewal, which generate progenitor cells which are irreversibly engaged for the production of one or more hematopoietic lines. Precise regulation of each step of differentiation is necessary in order to ensure a stable level of the different specialized cells as well as to offer a precise response to stimulations by stress. The regulations are usually the fact of interactions between cells, either by contact with the hematopoietic microenvironment or by the release of specific cytokines. Failure of the regulation systems leads either to cytopenia or uncontrolled cell proliferation, which can affect one or more lines depending on the nature of the lesion. Proliferation of several hematopoietic lines can lead to a myeloproliferative disease. Such diseases can be induced by retroviruses carrying oncogenes, such as the MPLV retrovirus, abbreviation for the expression "myeloproliferative leukemia virus," which is a retrovirus defective for its replication.

It is known that the murine MPLV retrovirus causes a severe hematological disorder in stem cells of adult mice of the majority of strains, characterized by dramatic proliferation and differentiation of several hematopoietic lines. The more acute aspect of this disease is the suppression of dependency in vitro with regard to growth factors, of the majority of the hematopoietic progenitor cells. The natural MPLV isolate is described as being a complex of two viral entities: a murine virus F-MuLV (Friend replication competent ecotropic murine leukemia virus) and a virus defective for its replication now designated by the term MPLV. The pseudotyping of the defective particles with other ecotropic or amphotropic MuLV viruses allows one to reproduce the initial disease. It has been shown that the proviral DNA of MPLV and the DNA of F-MuLV are structurally similar except in the region of the envelope.

The inventors have now identified a sequence of cellular origin transduced in a rearranged gene of the MPLV envelope and which proves to be preserved in the genome of mammals.

The identification of this sequence has revealed its importance in phenomena related to a myeloproliferative disease.

The invention relates therefore to a polypeptide which is capable of playing a part, when it is produced by an MPLV type virus, in the deregulations occurring in myeloproliferative diseases. The invention also relates to nucleotide sequences coding for this polypeptide.

The inventors have observed the very interesting fact that the protein sequence of the polypeptide of the invention has pronounced similarities with certain growth factor amino acid sequences. The invention consequently allows one to identify the

mechanisms of the pathology connected with the infection by the MPLV retrovirus and to propose means for detecting a pathology of the same type in humans and for treating it if necessary.

The invention therefore relates to a polypeptide characterized by the fact that it corresponds to the of amino acid chain which is designated by SEQ ID NO1 in the list of sequences, or by the fact that it includes the chain SEQ ID NO 1 or a fragment of this chain as long as the polypeptide meets at least one of the following conditions:

a) when it is produced from the genome of the MPLV retrovirus, it is capable of bringing about and/or promoting, in vitro or in vivo, proliferation of the hematopoietic cell lines,

b) in vitro or in vivo, it is involved in cell differentiation of hematopoietic cell lines, when it is produced from the genome of the MPLV retrovirus,

c) it is capable, in vivo, of being involved in a hematopoietic growth factor receptor function, either with regard to the binding of a ligand, or with regard to transmission of a signal,

d) it is recognized by antibodies directed against the chain amino acid chains represented by the sequence SEQ ID NO1,

or else by the fact that it is an amino acid sequence which is at least 80% homologous and preferably 88% homologous with the fragment represented by SEQ ID NO 2, which is contained in the amino acid chain SEQ ID NO 1.

The capacity of a given polypeptide for behaving like a growth factor receptor can be characterized by performing one of the following tests.

The invention allows one to detect the potential capacity of a polypeptide of the invention for behaving as a receptor and binding a ligand. One can, in effect, in the same way, study whether the expression of the polypeptide tested, previously modified by the MPLV virus for the nucleotide sequence coding for this polypeptide, integrated in the site usually containing the nucleotide sequence v-mpl, allows one to obtain proliferation in vitro of cells transformed with MPLV containing the polypeptide tested.

The conditions for performing this test are those used for the experiments reported hereafter, corresponding to the by MPLV of bone marrow cells in a culture.

In order to detect whether a nucleotide sequence coding for a polypeptide of the invention is capable of being involved in the transmission of a signal, one can modify MPLV in its site which usually contains the sequence v-mpl, using a nucleotide sequence of the invention, previously deprived of a chosen nucleotide fragment, of which one wishes to determine whether it is involved in the transmission of the signal. MPLV thus modified can be infected [sic], for example, with bone marrow cells according to what is described in the examples. One then sees whether the elimination of the nucleotide fragment in consideration above leads to the absence of cell proliferation.

Another test for detecting whether a polypeptide of the invention behaves as a receptor capable of both binding a chosen molecule and of transmitting a signal after the binding of this molecule, is the following:

cells not naturally expressing the polypeptide tested are transformed with the latter. The cells thus transformed are put

in the presence of a chosen molecule which is capable of behaving as a ligand, and one determines their capacity to respond to this molecule, particularly by seeing whether or not there is cell proliferation.

Particularly advantageous polypeptides corresponding to the definition of the invention given in the preceding are, for example, polypeptides containing an amino acid chain W S X W S, in which X is any amino acid, and X preferably corresponds to arginine or serine.

The sequence W S A W S corresponds to the fragment contained in v-mpl or in the equivalent sequence in mice, and the sequence W S S W S corresponds to the fragment h-mpl of the human cell gene.

Very interestingly, it was noted that the peptide W S X W S can be involved in the effect of differentiation in vivo of hematopoietic lines, which is observed during infection with an MPLV retrovirus.

The invention also relates to all the polypeptides which meet the preceding conditions, which are capable of inducing or participating in cell differentiation in vitro of hematopoietic lines.

Other preferred polypeptides according to the invention are the peptides characterized by the fact that they contain an amino acid chain corresponding to the chain identified in the list of sequences under reference SEQ ID NO 3.

Preferred polypeptides corresponding to the general definition above are the polypeptides corresponding to one of the sequences identified under the following references SEQ ID NO 2,

SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11.

Another polypeptide according to the invention is characterized by the fact that it is coded by a nucleotide sequence capable of hybridizing under stringent conditions with the MPLV specific probes corresponding to the fragments SACI-PSTI and PSTI-PSTI with 300 bp each, represented in Figure 1.

These probes can be DNA or RNA.

Also in the scope of the invention is a polypeptide coded by a nucleotide sequence capable of hybridizing with one of the preceding probes.

The invention also relates to a polypeptide included in the preceding definitions, characterized by the fact that it corresponds to one of the amino acid chains identified in the list of the sequences, under the following references SEQ ID NO 12, SEQ ID NO 13.

The invention moreover relates to a growth factor receptor, characterized by the fact that it contains the amino acid sequence identified by SEQ ID NO 1.

The invention also relates to a soluble form of the receptor particularly containing the extracellular region represented by the polypeptide sequence designated by SEQ ID NO 7 in humans or SEQ ID NO 10 in mice.

Also part of the invention are fusion proteins, characterized by the fact that they contain a polypeptide of the invention in association with a well defined amino acid chain, particularly with gp70 of MPLV, in particular by the fact that this pertains to the amino acid sequence SEQ ID NO 14.

Other fusion proteins can be obtained with the protein coded by the gene gag or with viral glycoproteins, for example, other than the retrovirus, particularly VSV glycoprotein.

The invention also relates to a nucleotide sequence characterized by the fact that it codes for a polypeptide described above.

Preferred nucleotide sequences are those which correspond to any one of the nucleotide sequences SEQ ID NO 15, NO 16, NO 17, NO 18, No 19, NO 20 or to a chain complementary to these sequences or else to a sequence capable of hybridizing with one of the sequences above under stringent conditions. Also included in the scope of the invention are the nucleotide sequences consisting of RNA and which correspond to the nucleotide chains described above.

The invention also relates to a recombinant cellular host, characterized by the fact that it contains a nucleotide sequence such as that given above. Preferably, the nucleotide sequence inserted in the cellular host is integrated in the genome of this host. Different cellular hosts can be chosen for the realization of the invention depending on the desired application. As an example, for the search for molecules which possibly have an affinity with the polypeptides of the invention, one can use cellular hosts such as bacteria, viruses, or phages, insect or mammal cells (COS, CHO), etc.

A particularly preferred cellular host is the MPLV virus. In particular, the invention relates to the MPLV virus which contains in its genome a nucleotide sequence coding for the peptide represented by the sequence SEQ ID NO 2 in the form of a fusion protein with the viral glycoprotein gp70.

The invention also relates to recombinant vectors, characterized by the fact that they contain a nucleotide sequence such as that described in the preceding, in site nonessential for its replication, under the control of the regulating sequences necessary for its expression in a determined cellular host.

Particularly suitable vectors for the execution of the invention are, for example, the vaccinia virus or the baculovirus.

Also part of the invention are nucleotide probes which contain at least 9 nucleotides and preferably 15 to 30 nucleotides, which are capable of hybridizing with a nucleotide sequence according to the invention, under stringent conditions, the probe being labeled if necessary.

The probes which are used can be specific to the nucleotide sequences of the invention or nonspecific if one is looking for a sequence belonging to a broader group.

Suitable labeling substances for the production of the probes of the invention are radioactive labels or else enzyme labels or any other suitable label.

The invention furthermore relates to polyclonal or monoclonal antibodies, characterized by the fact that they recognize one of the polypeptides of the invention.

Particularly advantageous antibodies are those directed against the extracellular region of the polypeptide coded by the gene c-mpl.

These antibodies are obtained according to conventional antibody production methods. In particular, for the preparation of monoclonal antibodies, one will use the method of Kohler and Milstein; in particular, one prepares monoclonal antibodies by

cell fusion between myeloma cells and spleen cells of mice previously immunized with a polypeptide according to the invention, according to the conventional process.

The identification by the inventors of the MPLV virus polypeptide responsible for the myeloproliferative disease allows for the development of means of detection in vitro of an abnormal expression of a polypeptide described in the preceding pages, which is indicative of a pathological condition.

For this purpose, the invention includes a process for the detection in vitro of an abnormal expression of a polypeptide of the invention, characterized by the fact that it includes:

- putting a biological sample capable of containing the polypeptide which is looked for, in contact with antibodies as defined in the preceding,

- detection of an immunological reaction of the antigen-antibody type.

"Abnormal expression" of the polypeptide is understood to mean any expression of abnormal quantitative level as well as any expression of a modified polypeptide whose functioning is altered.

Another process for the detection in vitro of abnormal expression of the polypeptide of the invention can be done by detection in vitro of abnormal expression of a nucleotide sequence coding for a peptide according to the invention. Such a process includes:

a) putting a biological sample capable of containing the nucleotide sequence studied, used as matrix, in contact with a nucleotide primer which is capable of hybridizing with the nucleotide sequence studied, in the presence of 4 triphosphate

different nucleoside, and a polymerization agent, under conditions of hybridization such that for each nucleotide sequence having hybridized with a primer, an elongation product of each primer, complementary to the matrix, is synthesized;

b) separation of the matrix and the elongation product obtained; the latter can then also behave as a matrix;

c) repetition of step a) so as to obtain a detectable quantity of the nucleotide sequences which are looked for;

d) detection of the product of amplification of the nucleotide sequences.

Details for the implementation of the PCR technique are given, for example, in the application EP 0,200,362 and in the application EP 0,229,701.

The invention also relates to a kit for the detection in vitro of the abnormal expression of the polypeptides according to the invention, characterized by the fact that it includes polyclonal or monoclonal antibodies capable of reacting with said polypeptide, [and], if necessary, a reagent for the detection of the antigen-antibody immunological reaction.

Such a kit can be considered for the execution of different types of tests, particularly RIA or ELISA tests.

The invention also relates to a process for the detection of the affinity of a molecule for a polypeptide according to the invention, characterized by:

- putting the molecule tested in contact with a cellular host previously modified by a nucleotide sequence according to the invention, under conditions allowing for the expression of this sequence, so as to obtain a polypeptide according to the

invention which has at least one site capable of interacting with the molecule tested exposed at the surface of the cellular host,

- detection of the formation of a complex between the molecule tested and the polypeptide.

The invention also relates to a drug, characterized by the fact that it contains a polypeptide corresponding to the preceding definitions, in soluble form, in combination with an acceptable pharmaceutical vehicle. Such a drug can be used to remedy the abnormal production of the polypeptides of the invention in the cells of a patient.

The drug according to the invention can act according to a competition reaction between the soluble form of a polypeptide present as active ingredient in this drug and the polypeptides present in an abnormal manner in the patient being treated.

Other characteristics and advantages of the invention appear in the figures and in the examples which follow.

Figure 1: (A) Restriction map by the restriction endonucleases of the clone MPLV 107 and of the clone F-MuLV 57. The restriction sites are given for the following enzymes: B: BamHI, C: ClaI, E: EcoRI, H: HindIII, K: KpnI, P: PstI, Pv: PvuII, SI: SacI, SII: SacII, X: XbaI.. The black frame corresponds to the MPLV specific region. The two probes derived from the MPLV envelope are indicated by lines under the restriction map.

(B) Demonstration of the specificity of the probes derived from the MPLV envelope. The cells NIH 3T3 infected with the amphotropic pseudotype 4070 of MPLV are used as source of poly A+ RNA (5 μg, lines A), the clone F-MuLV 57 (1 μg, lines B), and the MPLV-infected nonproducing clone 2 of Mus dunni, by MPLV (15 μg,

lines C). The Northern blotting prints are hybridized with the probe E57BS derived from the envelope of F-MuLV obtained, which is labeled according to the nick translation technique (sample No. 1), or with the two RNA probes derived from the envelope of MPLV, SacI-PstI (sample No. 2) and PstI-PstI (sample No. 3). The lines M represent the DNA lambda/HindIII molecular weight label. The black arrow and the light arrows indicate the positions of the genomic and subgenomic RNAs of the envelope of F-MuLV and MPLV, respectively.

Figure 2: (A) Diagrammatic representation of the rearranged env region of MPLV. The hatched frame represents mpl, the light frames represent the env sequences of F-MuLV. The asterisk in the MPL region indicates the stop codon.

(B) Amino acid sequence deduced from the env region of MPLV. The reading frame of 284 amino acids is given. Amino acids 1 to 100 correspond to the sequences derived from the env gene of F-MuLV. The 184 specific amino acids of mpl are framed. The heads of the arrows indicate the junction of the two portions of the envelope of F-MuLV and the junction with the mpl specific sequence, respectively. The asterisks indicate the potential sites of N-glycosylation (Asn-X-Ser/Thr). The underlined sequences represent the signal peptide of gp70 and the hydrophobic transmembrane region is written in bold-face.

Figure 3: Zoo blot

10 µg of high molecular weight DNA, digested by ECORI, of ICFW mice (line 1) and of Mus spretus (line 2), of rats (line 3), of minks (line 4), of cows (line 5), of dogs (line 6), of humans (line 7) were hybridized with the RNA probes SacI-PstI and PstI-PstI under very stringent hybridization conditions and washed

according to the protocols detailed in the "experimental procedures" part.

Figure 4: Expression of c-mpl in different mouse organs.

The Northern blot imprints of poly A+ RNA (5 µg) of mouse brain (line 1), of liver (line 2), of salivary gland (line 3), of spleen (line 4), of kidney (line 5), of testicle (line 6), of thymus (line 7), or of fetal liver (line 8) hybridized with the RNA probes SacI-PstI and PstI-PstI. The conditions of hybridization and washing are those described in the "experimental procedures" part.

Figure 5: Establishment in vitro of the cell lines infected by MPLV.

The normal C57BL/6 mouse bone marrow cells were infected in vitro with MPLV free of auxiliary viruses. The circles represent the average values + standard deviation of the nonadherent cells for five infected cultures (black circles) or five control cultures (white circles). The transfers of the nonadherent cell populations are indicted in dotted lines.

Figure 6: Southern analysis of the proviral integration sites in the cultures infected by MPLV. The DNAs prepared 5, 21, 97 days after the infection were digested with EcoRI and the prints were hybridized with the RNA probes of v-mpl. On day 97, L159 contained myeloblasts, L173 mastocytes, L223 megacaryocytes and erythrocytes. The arrow indicates c-mpl.

Figure 7: Comparison of the sequences of amino acids of the extracellular region of v-mpl with that of the hematopoietic cytokine receptors.

The extracellular regions of the murine IL-3 receptor, or the murine EPO receptor, of the murine IL-4 receptor, of the ß

chain of the IL-2 receptor, of the human IL-6 receptor, and of the human and murine IL-7 receptors were aligned with v-mpl. The conserved amino acid residues are framed. The consensus sequence is that which was described by Itoh et al., 1990 (Science, Vol. 247, pp. 324-327).

Examples

Molecular cloning of the MPLV provirus

The following experiments relate to the identification and characterization of the molecular rearrangements occurring in the envelope of the MPLV retrovirus.

In order to characterize the rearranged region of the env gene, a cDNA bank was prepared using the poly A+ RNAs of the NIH 3T3 cells affected in a productive manner with the MPLV amphotropic pseudotype. The cDNA clones containing the complete env region of MPLV were obtained and two MPLV specific probes were prepared: these are the fragments SacI-PstI and PstI-PstI, each with 300 bp (Figure 1A). The specificity of these two probes for MPLV is represented in Figure 1B. They recognize the genomic RNAs (7.4 kb) and subgenomic RNAs obtained by splicing (2.4 kb) but not hybridizing with F-MuLV or with amphotropic RNAs.

In order to clone a biologically active MPLV provirus, a genome bank was prepared from clones of nonproducing Mus dunni cells, containing a single copy of the MPLV provirus (Penciolelli et al., 1987). Among the 1.5 x 10^6 recombinant phages screened

with the two MPLV specific probes, a single clone was obtained (MPLV 107). Restriction analyses showed that this clone contains the complete genome of MPLV with the exception of the LTR 3′ part (Figure 1A).

In order to demonstrate that this molecular entity was responsible for the characteristics of the acute myeloproliferative disease caused by MPLV, a complete provirus was constructed by ligation of MPLV 107 with F-MuLV 57 3′ LTR. The resulting construct (MPLV3) was cotransfected with the DNA of the auxiliary virus F-MuLV in the NIH 3T3 cells according to a molar ratio of 10/1 (MPLV3/F-MuLV). After a few cellular passages the viral supernatant was injected intravenously in young adult DBA/2 mice known for their resistance to early erythroleukemia induced by F-MULV (Ruscetti et al., 1981, J. Exp. Med. 154, 907-920).

The animals inoculated with the supernatant of the cultures transfected with the DNA of F-MuLV alone were healthy 6 months after the infection. In contrast, all the mice inoculated with the supernatant of the cultures transfected with the DNAs of MPLV3 and F-MuLV rapidly developed splenomegaly, hyperleukocytosis, and polycythemia and died two months after the inoculation. The progenitor cells of the sick animals were then examined in vitro in order to demonstrate their needs in terms of hematopoietic growth factor. 100% of the late progenitor cells of the red blood cells (erythroid colony forming units (CFU-E) formed colonies of erythrocytes with hemoglobin without addition of erythropoietin (EPO), while 62% of the colony forming cells CFU-C in the spleen and 30% of the CFU-C in the bone marrow proliferated and differentiated without exogenous addition of

colony stimulating factors. They led to mature colonies of granulocytes, monocytes, megacaryocytes, erythrocytes, and to multipotent colonies containing different cell lines.

Thus, the clone MPLV3 was biologically active and its properties could not be distinguished from those of the original MPLV isolate.

Sequence and structure of the env region of MPLV

The rearranged env genes of the cDNAs of MPLV and of the genomic clone were sequenced and prove to be identical. The nucleotide sequence was analyzed, showing that the env gene of MPLV includes sequences derived from the env gene of F-MuLV and nonviral sequences. As illustrated in Figure 2A, two deletions appeared in the env gene of F-MuLV: the first between positions 5969 and 6505 and the second between the positions 6615 and 7513 (Koch et al., 1983, J. Virol. 45, 1-9). The env gene of MPLV is therefore a complex region composed between the 5′ and 3′ ends of 191 bp of the 5′ end of the env gene of F-MuLV (until position 5969) followed by 110 bp of the central region of the env gene of F-MuLV (between positions 6506 and 6615), then by a nonviral region of 623 nucleotides, and finally the 3′ part of the protein 15E of F-MuLV (starting from position 7513).

The env gene of MPLV has an open reading frame of 284 amino acids starting from the ATG initiation codon of the gp70 and ending with a termination codon TAG inside the MPLV specific sequence (Figure 2B), and potentially codes for a fusion protein env with a molecular weight of 31 kd. The env-vmpl fusion

protein includes 64 amino acids from the NH_2-terminal part of the gp70 of F-MuLV including the signal peptide, 36 amino acids of the central region of the env gene of F-MuLV, and 184 MPLV specific amino acids.

A hydrophobicity curve (Kyte et al., 1982, J. Mol. Biol., 157, 105-132) of the amino acid sequence of the product of the env gene of MPLV revealed in addition to the 34 hydrophobic amino acids of the signal peptide of gp70, that the MPLV specific region contained a region of 32 uncharged amino acids extending from the amino acid Ile 143 to the amino acid Leu 165, which may correspond to a membrane region. The natural env protein of MPLV would thus consist of an extracellular region of 109 amino acids with a potential glycosylation site, a transmembrane region of 22 amino acids, and an intracytoplasmic region of 119 amino acids without sequence for kinase activity (Hanks et al., 1988, Science, Vol. 241, pp. 42-52).

A search through the EMBL data (nucleotides and proteins) indicates that the MPLV specific sequence, designated by v-mpl, does not correspond to a gene which has been identified up to now.

env region of MPLV containing a unique cellular sequence highly conserved in mammals

The presence of a possible c-mpl locus in mice, rats, minks, cows, dogs, and humans was looked for. The hybridization of the DNA digested by EcoRI with the two RNA probes of v-mpl allowed one to detect clear bands under stringent hybridization

conditions, indicating the presence of a cellular counterpart (c-mpl) to the sequence v-mpl in the 6 species tested (Figure 3).

One then looked for the expression of c-mpl in different mouse tissues. Northern blot prints of poly A+ RNA prepared from fetal liver and from different adult mouse organs hybridized with v-mpl RNA probes. As shown in Figure 4, a single band of mRNA of 3.0 kb could be detected in the adult spleen (line 4) and in the fetal liver (line 8). A similar transcript was also present in the bone marrow. In contrast, no transcript was detected in the brain, the liver, the salivary glands, the kidney, the testicles, or the thymus of adult mice.

The leukemogenic properties of MPLV can thus be attributed to the presence of a new oncogene, v-mpl, transduced from cellular sequences conserved in the phylogeny of mammals and transcribed in the normal murine hematopoietic tissues.

MPLV transforms hematopoietic progenitors in vitro

In order to determine whether MPLV could directly transform hematopoietic cells and in order to analyze the target cells of the virus, bone marrow cells were infected in vitro with MPLV free of auxiliary virus, obtained in a packaging ψ-CRE cell line (Danos and Mulligan, 1988, P.N.A.S. USA 85, 6460-6464). The test was performed in an agarose medium with a low cell concentration in order to avoid stimulating the formation of colonies by the endogenous factors secreted by the accessory cells. In repeated experiments, only a few autonomous colonies were detected. Nevertheless, when the infection was done with bone marrow cells enriched in immature progenitors made to divide by pretreatment

of the mice with 5-fluorouracil (5-FU) (Hodgson and Bradley, 1979, Nature, Vol. 281, pp. 381-382), colonies developed spontaneously in significant numbers. Approximately half were colonies of a single line, such as colonies of megacaryocytes or granulocytes or erythroid colonies. The other colonies were mixed colonies of which approximately 20% had three or more than three lines of differentiation. Experiments of subculturing colonies containing one or two lines of differentiation did not lead to the production of secondary colonies, which indicates that these colonies resulted from progenitors irreversibly engaged in differentiation. In contrast, more than 65% of the colonies containing several lines of differentiation (12/18) produced a variable number of secondary colonies (7 to 286), expressing one or two lines of differentiation but also macroscopic colonies in which at least three lines were present. Certain ones of these colonies (3/18) produced mixed tertiary colonies. This indicates that MPLV is capable of promoting the proliferation and terminal differentiation both of multipotent stem cells and of progenitor cells already engaged in differentiation.

MPLV immortalizes bone marrow cells in culture and induces their differentiation

When bone marrow cells infected with MPLV and coming from normal mice or from mice pretreated with 5 FU were cultured, an increase of the percentage by 10 to 20 times of the frequency of progenitor cells was observed at the same time as an increase of colonies independent of growth factors. The change evolution of

such cultures is shown in Figure 5. While in the uninfected cultures, the cells have a low growth, the cultures infected with MPLV contain nonadherent cells which divide rapidly and which can be transferred into new bottles devoid of adherent nutritive stromal cells. The cells continued to proliferate forming permanent cell lines growing in suspension and containing erythroblasts in terminal phase of differentiation, megacaryocytes, and polymorphonuclear leukocytes in association with immature blast cells. When these cells are grown in semisolid medium, different types of autonomous colonies containing mature cells develop.

Four to six weeks after infection, the majority of the cell lines evolved towards a more limited phenotype which remained apparently stable during months of continuous culture. A morphological examination of the cells in suspension and of the colonies obtained in the semisolid medium showed that among the 24 lines, one line remained multipotential, five contained mature megacaryocytes and erythroblasts with hemoglobin, five were composed of megacaryocyte cells, five were composed of mastocytes, four were composed of myelomonocyte cells, two contained erythroblast cells in differentiation phase, and two corresponded to immature blast cells. Experiments took place to determine whether these cultures were of the polyclonal or monoclonal type. This results in the fact that these permanent cell lines are obtained from the growth of one or a few multipotential strains infected with MPLV, in which the restriction of the differentiation capacities occurs in a later stage. The malignant character of the cell lines was

demonstrated by subcutaneous injection of 2 x 10^6 cells in syngeneic mice irradiated with a sublethal dose (5 Gy) or in nude mice. No tumor developed at the site of the inoculation when the cells coming from a culture less than 4 months old were grafted, but 6 of the 10 cells lines inoculated after more than 7 months produced hematopoietic tumors of the nature of these grafted cell lines after a latency period of approximately 30 days.

In order to find out whether the autonomous growth of the cells resulted from the production of a growth factor, 10 times concentrated conditioned media were tested with different cell lines based on the indicator cell line FDC-P1 (Dexter et al., 1980, J. Exp. Med. 152, 1036-1047). No incorporation of ^{3}H-thymidine could be detected. The continuous growth was therefore not sustained by the secretion of IL3 or GM-CSF. Moreover, Northern blot analyses did not reveal the mRNA of IL3, of GM-CSF, of G-CSF or of EPO in twelve cell lines examined, with the exception of one cell line which expressed the G-CSF mRNA.

Thus, these observations indicate that MPLV alone is capable of directly transforming hematopoietic progenitors which are engaged in differentiation and are multipotential, and leads to the rapid emergence of different immortalized cell lines, independent of growth factors, which keep the capacity to spontaneously differentiate.

The experiments described in the preceding showed that the pathogenicity of MPLV was not due to major modifications in its LTR part. Analysis of the sequence of the <u>env</u> region of MPLV showed that the <u>env</u> gene of MPLV did not contain sequences related to MCF virus sequences but that a 1.5 kb sequence of the

F-MuLV envelope was deleted and replaced by a new 0.7 kb nonviral sequence which was not homologous with known genes. This new sequence, v-mpl, is of cellular origin and is preserved in mammals, including humans. The protooncogene c-mpl is transcribed in the form of a 3.0 kb mRNA in the adult mouse spleen and in the bone marrow and in the fetal liver. The chromosomal location of c-mpl is chromosome 4 in mice, and human chromosome 1-p34.

The polypeptide env-mpl has the general characteristics of a transmembrane protein: it contains the gp70 signal peptide at its N-terminal part and a single transmembrane region, indicating that the N-terminal part of the molecule is extracellular and the C-terminal part is intracytoplasmic. The amino acid sequence of the extracellular region of the v-mpl protein has similarities with the recently cloned cytokine hematopoietic receptors such as the ß chain of IL2R, IL3R, IL4R, IL6R, IL7R, GM-CSFR, G-CFSR, EPO-R, as well as with the prolactin receptor. Thus, this sequence contains a W S X W S unit in the extracellular region near the transmembrane region and does not contain a consensus sequence for protein kinase activity in the intracytoplasmic region. It was also observed that the cytoplasmic region of v-mpl contains many prolines (14/19, 12%) and serines (13/119, 11%) as is the case of other receptors. This results in the fact that MPLV has transduced a truncated form of a hematopoietic growth factor receptor. The expression of the c-mpl gene observed only in the spleen cells, the bone marrow, and the fetal liver, confirms this hypothesis.

Experimental procedures

Cells, viruses, and mice

NIH 3T3 and Mus dunni cells were used. The isolation of MPLV non-producer clone 2 of Mus dunni was described in the publication of Penciolleli et al., 1987 (J. Virol. 61, 579-583). The amphotropic MPLV pseudotype was obtained by superinfecting clone 2 of Mus dunni with the amphotropic auxiliary virus 4070 A (Chattopadhyay et al., 1981; J. Virol. 39, 777-791).

DBA/2J, C57BL/6J and nude mice were obtained from Iffa Credo (l'Arbresle, France) and raised under conditions devoid of pathogenic agents. Animals 6 to 8 weeks old were used in all the experiments.

Preparation of the RNA and Northern Blot analysis

The total RNA was purified according to the method using guanidine thiocyanate/CsCl (Chirgwin et al., 1979; Biochemistry 18, 5294-5299), and the poly A+ RNAs were selected by oligo dT cellulose column chromatography.

For the Northern Blot analysis, 5 μg of poly A+ RNA were denatured in a glyoxal buffer according to McMaster and Carmichael, 1977 (Proc. Natl. Acad. Sci. USA, 74, 4835-4838). Electrophoresis took place in 1.1% agarose gels in 10 mM Na/Na_2 phosphate buffer. The RNA transfers were done on Hybond C-extra nitrocellulose (Amersham) as described by Thomas, 1980 (Proc. Natl. Acad. Sci. USA 77, 5201-5205). The membranes were

prehybridized for 5 h at 55°C in 50% formamide, 4X SSC, 0.05M Na/Na_2 phosphate, 1X Denhardt, 500 µg/mL yeast tRNA, and 250 µg/mL herring sperm DNA. 10^7 cpm of a ^{32}P-labeled RNA probe was added, and hybridization occurred for 40 h at 55°C. The membranes were washed twice for 5 min at room temperature in 2X SSC-0.1% SDS, twice for 30 min at 65°C in 2X SSC-0.1% SDS, and twice for 30 min at 65°C in 0.1X SSC-0.1% SDS.

Southern Blot analysis

The DNAs were digested with appropriate restriction endonucleases according to the conditions indicated by the manufacturer, and deposited on 0.8% agarose gel. After electrophoresis, the DNAs were transferred onto nitrocellulose membranes according to the Southern method (1975; J. Mol. Biol. 98, 503-518). The membranes were hybridized with 10^7 cpm of ^{32}P-labeled probe under conditions described for the Northern Blot.

Construction of the bank of cDNA of the MPLV envelope

The cDNA was synthesized from the poly A+ RNA prepared from NIH 3T3 cells in exponential growth phase, infected in a productive manner with MPLV pseudotyped by the auxiliary amphotropic virus 4070 A (MPLV containing the envelope of the virus 4070 A), using the cDNA synthesis kit of Amersham. cDNAs were bound at the free end to a dephosphorylated vector pSPT18 digested with SmaI in the presence of DNA T4 ligase.

Competent LM 1035 bacteria were transformed and spread on dishes of agar containing ampicillin. Colonies containing the recombinant plasmids were transferred onto filters of nitrocellulose. The identification of the clones containing the cDNA of the envelope (*env*) of MPLV was done by hybridization in situ as described by Sambrook et al., 1989 (Cold Spring Harbor laboratory Press), with a probe E57BS (Moreau-Gachelin et al. 1983 Biochimie 65, 259-266), labeled with ^{32}P by the "nick translation" technique.

Construction of the MPLV genome bank

The high molecular weight DNA was extracted according to Souyri et al. 1983 (Proc. Natl. Acad. Sci. USA 80, 6676-6679) from the clone MPLV nonproducer 2 of *Mus dunni* and partially digested with *Sau*3A restriction endonuclease. The DNA fragments (10 to 15 Kb) were purified by centrifugation on sucrose gradient and bound to *Bam*H1 arms of the bacteriophage EMBL3 after packaging (Stratagene). After encapsulation in vitro (Gigapack, Stratagene), the recombinant phages containing the MPLV DNA were identified according to the technique of Benton et al., 1977 (Science 196, 180-182) by hybridization with MPLV specific RNA probes. The filters were prehybridized for 5 h at 42°C in 50% formamide, 5X SSC, 5X Denhardt, 0.1% SDS, 50 mM of Na/Na_2 phosphate pH 6.5, and 250 µg/mL of herring sperm DNA (2 mL per filter). The hybridization with ^{32}P-labeled MPLV RNA probes was carried out for 20 h at 42°C in 50% formamide, 5X SSC, 1X Denhardt, 0.1% SDS, 50 mM of Na/Na_2 phosphate pH 6.5, and 250

μg/mL of herring sperm [DNA] (1 mL of buffer and 2 x 10^6 cpm of RNA probe per filter). The filters were washed twice for 10 min at room temperature in 2X SSC-0.1% SDS, for 30 min with 2X SSC-0.1% SDS, and twice for 30 min with 0.2X SSC-0.1% SDS, each time at 65°C.

Sequencing of the DNA

The DNA sequence was obtained using the chain termination dideoxy method (Sanger at al., 1977; Proc. Natl. Acad. Sci. USA 74, 5463-5467) modified for the use of DNA T7 polymerase (Sequenase USB). The samples were denatured for 2 min at 75°C and placed on a denaturing acrylamide gel (6% acrylamide, 8M urea, 1X TBE).

The analysis of the sequences, the comparison of the nucleotide and protein sequences between mpl and the genes included in the EMBL bank were done using the FASTP programs (Lipman et al., 1985, Science 227, 1435-1441) and PC-GENE (Intelligenetics Inc. and Genofit SA).

Infection in vitro of hematopoietic cells and establishment of cell lines

Cellular clones producing MPLV but devoid of auxiliary virus were produced by cotransfection of packaging ψ-CRE cells, with the plasmid pSV2 Neo and an excess by approximately 10 times of the plasmid pMPLV3. After selection and isolation of G418-resistant clones (Gibco BRL), the MPLV producing clones were selected by prints of the whole cells according to Wendling et al., 1989 (Leukemia 3, 475-480). The prints of the virus

purified from the supernatants were used to select a clone producing a high titer of virus. 5 million normal bone marrow cells or 1.5 x 10^6 cells of male adult C57BL/6 mice pretreated with 5-fluorouracil (150 mg/kg of body weight, 4 days before) were suspended in 1 mL of infectious supernatant. Incubation was done for 2 h at 37°C in an atmosphere containing 5% CO_2 in air. The cells were than placed in a semisolid medium or grown a concentration of 2.5 x 10^6 cells/mL in 25 cm^2 culture bottles containing 8 mL of Dulbecco's complete medium modified according to Iscove (IMDM) with 20% heat-indicated fetal calf serum (Flow Laboratories). After 10 to 12 days, nonadherent cells were recovered and transferred in a concentration of 2.5 x 10^5 cells/mL into new bottles containing new medium. Then, the cells were passed every 4 to 7 days, or more frequently, depending on the level of cell growth.

Tests on the progenitor cells

For the CFU-E colonies, the cells were seeded in a coagulated plasma culture system as described by McLeod et al., 1978 (M.J. Murphy, ed., Springer Verlag, New York, pp. 31-35). An appropriate number of cells was distributed in a volume of 0.1 mL with or without 0.25 U/mL Epo (erythropoietin) (Stage 1 human EPO, specific activity 1000 U/mg; Terry Fox Laboratory, Vancouver, Canada). The cultures were collected on day 2. The colonies containing at least 8 erythroblasts positive with benzidine were listed as CFU-E colonies.

For the CFU-C colonies, the tests were done in 0.5 mL of agarose (Seaplaque agarose, FMC) in cultures on Linbro plates (CT-CV 96) according to the aforementioned method of McLeod et

al., 1978. For the control cultures, the formation of colonies was stimulated to the maximum by addition of 5% (v/v) of a conditioned medium prepared from spleen cells stimulated with a mitogenic agent (PWMSCM) and 1 U/mL Epo. The cultures were incubated in a moisture saturated atmosphere containing 5% CO_2. After 7 days of incubation, the cultures were withdrawn from the wells, placed on slides and fixed in a phosphate buffer containing 5% glutaraldehyde (pH 7) stained with benzidine, myeloperoxidase or acetylcholinesterase then with hematoxylin, in order to determine the cell composition of each colony.

* * *

Claims

1. A polypeptide, characterized by the fact that it corresponds to the amino acid chain designated by SEQ ID NO1 in the list of sequences, or by the fact that it includes the chain SEQ ID NO 1 or a fragment of this chain as long as the polypeptide meets at least one of the following conditions:

a) when it is produced from the genome of the MPLV retrovirus, it is capable of bringing about and/or promoting, in vitro and in vivo, the proliferation of the hematopoietic cell lines,

b) in vitro or in vivo, it is involved in cell differentiation of hematopoietic cell lines, when it is produced from the genome of the MPLV retrovirus,

c) it is capable, in vivo, of being involved in a hematopoietic growth factor receptor function, either with regard to the binding of a ligand, or with regard to transmission of a signal,

d) it is recognized by antibodies directed against the amino acid chain represented by the sequence SEQ ID NO1,
or else by the fact that it is an amino acid sequence at least 80% homologous and preferably 88% homologous with the fragment represented by SEQ ID NO 2, which is contained in the amino acid chain SEQ ID NO 1.

2. A polypeptide according to Claim 1, characterized by the fact that it contains an amino acid chain WSXWS, in which X is any amino acid, and preferably corresponds to arginine or serine.

3. A polypeptide according to Claim 1, characterized by the fact that it is a fragment identified under the reference SEQ ID NO3.

4. A polypeptide according to any one of Claims 1 to 3, characterized by the fact that it corresponds to one of the amino acid chains identified under the following references: SEQ ID NO 2, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11.

5. A polypeptide according to any one of Claims 1 to 4, characterized by the fact that it is coded by a nucleotide sequence capable of hybridizing under stringent conditions with the probes SacI-PstI or PstI-PstI of the sequence identified by the reference SEQ ID NO 2 or else with one of these two probes.

6. A polypeptide according to any one of Claims 1 to 3, characterized by the fact that it corresponds to one of the amino acid chains identified under the following references: SEQ ID NO 12, SEQ ID NO 13.

7. A growth factor receptor, characterized by the fact that it contains the sequence SEQ ID NO 1 according to Claim 1 or by the fact that it contains a soluble form of this sequence.

8. A fusion protein, characterized by the fact that it contains a polypeptide according to any one of Claims 1 to 5, in association with a known amino acid chain, particularly with gp70 of MPLV, in particular by the fact that this pertains to the amino acid sequence SEQ ID NO 14, or else with the protein coded by the gene gag or with an exogenous glycoprotein, for example, the glycoprotein of a virus other than a retrovirus, such as VSV.

9. A nucleotide sequence, characterized by the fact that it codes for a polypeptide according to any one of Claims 1 to 8.

10. A nucleotide sequence, characterized by the fact that it corresponds to any one of the nucleotide sequences SEQ ID NO 15, NO 16, NO 17, NO 18, No 19, NO 20 or to a chain complementary to these sequences, or else to a sequence capable of hybridizing with one of the sequences above under stringent conditions.

11. A recombinant cellular host, characterized by the fact that it contains a nucleotide sequence according to either Claim 9 or 10, integrated in its genome.

12. A cellular host according to Claim 11, characterized by the fact that it is a virus, particularly the MPLV virus, a bacterium, an insect or mammal cell such as COS or CHO.

13. A recombinant vector, characterized by the fact that it contains a nucleotide sequence according to either Claim 9 or 10, under the control of the regulatory sequences necessary for its expression in a known cellular host; this vector can for example be the vaccinia virus [or] baculovirus.

14. A nucleotide probe which contains at least 9 nucleotides, which is capable of hybridizing under stringent conditions with a nucleotide sequence according to either Claim 9 or 10; this probe is labeled if necessary.

15. Polyclonal or monoclonal antibodies, characterized by the fact that they recognize an amino acid chain according to any one of Claims 1 to 6.

16. A process for the detection in vitro of an abnormal expression of a polypeptide according to any one of Claims 1 to 6, which includes:

- putting a biological sample capable of containing the polypeptide studied in contact with antibodies according to Claim 16,

- detection of an immunological reaction of the antigen-antibody type.

17. A process for the detection in vitro of abnormal expression of a nucleotide sequence according to either Claim 9 or 10, which includes:

a) putting a biological sample capable of containing the nucleotide sequence studied used as matrix, in contact with a nucleotide primer which is capable of hybridizing with the nucleotide sequence which is looked for, in the presence of 4 different triphosphate nucleosides and a polymerization agent, under conditions of hybridization such that for each nucleotide sequence having hybridized with a primer, a product of elongation of each primer, complementary to the matrix is synthesized;

b) separation of the matrix and the elongation product obtained; the latter can then also behave as a matrix;

c) repetition of step a) so as to obtain a detectable quantity of the nucleotide sequences which are looked for;

d) detection of the product of amplification of the nucleotide sequences.

18. A process for the detection of the affinity of a molecule for a polypeptide according any one of Claims 1 to 5, characterized by:

- putting the molecule tested in contact with a cellular host previously modified by a nucleotide sequence according to either Claim 9 or 10, under conditions allowing for the expression of this sequence, so as to obtain a polypeptide according to any one of Claims 1 to 5 which has at least one site capable of interacting with the molecule tested, exposed at the surface of the cellular host,

- detection of the formation of a complex between the molecule tested and the polypeptide.

19. A drug, characterized by the fact that it contains a polypeptide according to any one of Claims 1 to 6 in soluble form, in combination with an acceptable pharmaceutical vehicle.